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<b>(21) International Application Number:</b> PCT/US97/04095 <b>(22) International Filing Date:</b> 18 March 1997 (18.03.97) <b>(30) Priority Data:</b> 08/617,069                      18 March 1996 (18.03.96)                      US <b>(71) Applicant:</b> THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104 (US). <b>(72) Inventors:</b> GARCIA, Andres, J.; University of Pennsylvania, Dept. Bioengineering, 3320 Smith Walk, Philadelphia, PA 19104 (US). DUCHEYNE, Paul; 763 Applegate Lane, Rosemont, PA 19010 (US). BOETTIGER, David; University of Pennsylvania, Dept. of Microbiology, 209 Johnson Pavilion, Philadelphia, PA 19104 (US). <b>(74) Agents:</b> ELDERKIN, Dianne, B. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris L.L.P., 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> BIOACTIVE MATERIAL SUBSTRATE FOR ENHANCED CELLULAR ATTACHMENT AND FUNCTION		
<b>(57) Abstract</b>  <p>Novel non-crystalline, porous bioactive glass and ceramic materials that permit the <i>in vitro</i> formation of bone tissue when exposed to a tissue culture medium and inoculated with cells are disclosed. The present invention also discloses bioactive glass materials treated such that when the glass is in contact with anchorage-dependent cells, there is enhanced cell attachment and cell function. Expeditious tissue growth occurs <i>in vitro</i> or <i>in vivo</i>. The glass material disclosed is preferably formed from SiO<sub>2</sub>, CaO, Na<sub>2</sub>O and P<sub>2</sub>O<sub>5</sub>, although other oxides may be included, and is most preferably prepared by melting the constituents, cooling and pulverizing the resulting glass, and then forming and hot pressing the powder. The glass of the present invention may be formed to produce templates that are useful for various indications, as well as granules that may be formed into a paste.</p>		

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## BIOACTIVE MATERIAL SUBSTRATE FOR ENHANCED CELLULAR ATTACHMENT AND FUNCTION

This is a continuation-in-part of U.S. Application  
Serial No. 08/278,579, filed July 21, 1994, pending, which is  
5 a continuation of U.S. Application Serial No. 07/929,104,  
filed August 13, 1992, abandoned.

The present invention relates to the synthesis of  
bioactive ceramic templates for optimum *in vitro* formation of  
bone and bone-like tissue, and the use of bioactive  
10 substrates for the enhanced cellular attachment and function  
of anchorage-dependent cells. The fundamental mechanisms by  
which material surfaces elicit the *in vitro* response from  
bone cells are optimized, thereby leading to high formation  
rates of extracellular material with typical characteristics  
15 of bone tissue.

## BACKGROUND OF THE INVENTION

Materials were first used to provide structural  
support during healing of bones, or to replace damaged or  
diseased bone tissue. Historically, the most important  
20 material selection criterion was inertness. It was believed  
that the implant material should only provoke the slightest  
reactions in the body. It is important to realize, though,  
that no matter how chemically inert a material may be, it  
always provokes a reaction upon implantation. The intensity  
25 of the reaction depends not only on the surface and bulk  
properties of the implant material, but also on the trauma at  
the time of surgery, the site of implantation, and the  
relative motion at the tissue-implant interface. This  
observation has prompted the use of "bioactive" materials

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instead of so-called inert materials. The implication is that a bioactive material must provoke a beneficial tissue response, specifically it must elicit the formation of the normal tissue at its surface and create an interface that promotes long functional life. Whereas the field of calcified tissue reconstruction has achieved this goal, this advance is not an end state, but merely a stepping stone for an even more ambitious goal: the creation of materials that are capable of serving as templates for *in vitro* bone tissue formation. This is part of the true future of biomaterials: creating materials that, once inserted into the body, regenerate tissues rather than replace them.

Cell culture studies with osteoprogenitor cells or cells of osteoblastic phenotype have been performed, but never achieved acceptable results. Some of these prior studies did not seek the optimization *per se* of extracellular material synthesis. Some prior studies used osteoprogenitor cells present in bone marrow extracts. Regardless of whether focus is placed on the determination of osteoblastic phenotype expression or elsewhere, these results can be used to determine whether one of the resultant phenomena of osteoblastic phenotypic activity was extensive or not.

It is known to obtain bone marrow cells from the femora of young adult male Wistar rats by washing them out with  $\alpha$  MEM (minimal essential medium) supplemented with 15% fetal bovine serum, freshly prepared ascorbic acid, sodium  $\beta$ -glycerophosphate, dexamethasone (DEX) and antibiotics. See, Davies et al. "Early extracellular matrix synthesis by bone cells," Bone-Biomaterials Workshop, J.E. Davies Ed., University of Toronto Press, (December 1990). A quantity of this cell suspension, e.g., 30 ml, containing cells from two femora, is aliquoted on to the material substrate. In a humidified 95% air - 5% CO<sub>2</sub> atmosphere the culture is maintained for a minimum of two weeks. It was shown that a calcified matrix of globular accretions, also containing sulphur, is formed. This layer was typical for reversal lines in bone tissue, the cementum layer, and was considered

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by the authors as evidence that the calcified layer is the result of the expression of the osteoblastic phenotype by the cultured cells. Subsequently, there was what was called "frank bone formation." Thus, matrix production can start  
5 within a time period of intermediate duration (17 days) by differentiating bone-derived cells *in vitro*. It has also been reported, however, that no calcified tissue formation has been obtained on porous ceramics. See Uchida et al. "Growth of bone marrow cells on porous ceramics *in vitro*," J.  
10 Biomed, Mat. Res. 21:1-10 (1987). The observation in the prior art with respect to the intrinsic capability of cells to deposit a cement-like line is in any event certainly correct. The cell culture method described above is derived from Maniatopoulos et al.'s "Bone formation *in vitro* by  
15 stromal cells obtained from bone marrow of young adult rats," Cell Tissue Res. 254:317-330 (1988), wherein this particular cellular activity was shown to be present in cultures without any tissue stimulating biomaterial.

The effect of porous calcium phosphate ceramic on  
20 growth and hormonal response of periosteal fibroblasts, osteoblasts, and chondrocytes has been disclosed by other workers. See, e.g., Cheng et al., "Growth of osteoblasts on porous calcium phosphate ceramic: an *in vitro* model for biocompatibility study," Biomaterials, 10, 63-67 (1989). As  
25 reported in this reference, the number of these cells increased 29-, 23- and 17-fold during a ten week time period. Osteoblasts retained their phenotypic expression by producing only Type I collagens. Previously, Cheng had shown that the phenotypic expression of canine chondrocytes had been  
30 retained up to 13 months when cultured on porous hydroxyapatite ceramic granules. See Cheng, "In vitro cartilage formation on porous hydroxyapatite ceramic granules," In Vitro Cellular & Developmental Biology, 21:6, 353-357 (1985). The elaboration of extracellular matrix  
35 reportedly started to appear at week one and increased throughout a thirteen month period.

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Still others have studied the attachment and subsequent growth of V79 cells in contact with various calcium phosphate ceramics and found that cell growth was markedly inhibited by hydroxyapatite, and slightly inhibited by tricalcium phosphate and glass ceramics. See Katsufumi et al., "The influence of calcium phosphate ceramics and glass ceramic on cultured cells and their surrounding media," J. Biomed Mat. Res., 24:1049-1066 (1989). Under conditions of phagocytosis of small bioactive ceramic powders, RNA transcription and protein synthesis of osteoblast populations have been stimulated. See Gregoire et al. "The influence of calcium phosphate biomaterials on human bone cell activities: An *in vitro* approach," J. Biomed Mat. Res. 24:165-177 (1990). This phenomenon has also been observed for phagocytosing fibroblasts. It has been suggested that the increase of <sup>3</sup>H-thymidine incorporation into DNA and the decrease of alkaline phosphatase activity probably resulted from secondary calcium messenger pathways. See Orly et al. "Effect of synthetic calcium phosphate on the <sup>3</sup>H-thymidine incorporation and alkaline phosphatase activity of human fibroblasts in culture," J. Biomed Mat. Res. 23:1433-1440 (1989). Another study by Puleo et al., "Osteoblast responses to orthopaedic implant materials *in vitro*," J. Biomed Mat. Res. 25:711-723 (1991), provided inconclusive results regarding osteoblast attachment, osteoblast proliferation and collagen-synthesis.

Another set of studies performed *in vivo*, documenting materials-dependent tissue response patterns are noteworthy. A series of experiments with porous hydroxyapatite and bone marrow cells was started by Ohgushi, Goldberg and Caplan and subsequently continued separately by Ohgushi and associates in Nara, Japan and Caplan and associates in Cleveland, Ohio (USA). See Ohgushi et al. "Heterotopic osteogenesis in porous ceramics induced by marrow cells," J. Ortho. Res., 7:568-578 (1989). These experiments demonstrate that the osteoprogenitor nature of the cells of a marrow cell suspension, implanted in heterotopic sites, are activated

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more readily when the suspension is infused into porous hydroxyapatite than when implanted by itself.

Finally, U.S. Patent No. 4,609,501 -- Caplan et al. discloses the stimulation of bone growth that includes the *in vitro* exposure of isogenic fibroblasts to a soluble bone protein capable of stimulating a chondrogenic response. The exposed cells are combined with a biodegradable carrier such as fibrin, although it is also suggested that the exposed cells may also be incubated with a prosthesis. A related Caplan et al. patent, U.S. Patent No. 4,609,551, discloses techniques for delivering the bone protein to anatomical sites, while U.S. Patent No. 4,608,199 also to Caplan et al. discloses processes for obtaining suitable bone protein.

The present invention is focused on substrate materials and shows that appropriate modification of the material used as the substrate can lead to major differences in amount and rate of tissue formation *in vitro*. It is thus an object of this invention to synthesize and treat bioactive materials which can serve as the ideal templates upon which life processes, for example, bone tissue formation, can thrive.

#### SUMMARY OF THE INVENTION

These and other objects are met by the synthesis of porous glass without producing significant crystallization and the conditioning of the glass surface such that cell attachment is enhanced and extensive extracellular matrix (ECM) formation can take place. Control of the bioactivity reactions to produce pH variation in solution within boundaries that do not kill the cells once seeded on the porous glass is also disclosed.

Generally, the present invention discloses a bioactive material surface treated to enhance bone cell attachment and activity when the material is placed in a tissue culture medium, such that when inoculated with cells, bone tissue forms *in vitro*. Preferably, the bioactive comprises a glass treated to control pH, most preferably to less than 7.6. In

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a preferred embodiment, the bioactive material is a non-crystalline glass consisting essentially of:  $\text{SiO}_2$ ;  $\text{CaO}$ ;  $\text{Na}_2\text{O}$ ; and  $\text{P}_2\text{O}_5$  having the following preferred composition: 45% by weight  $\text{SiO}_2$ ; 24.5% by weight  $\text{CaO}$ ; 24.5% by weight  $\text{Na}_2\text{O}$ ; and 5 6% by weight  $\text{P}_2\text{O}_5$ . Alternatively, in other preferred embodiments, the bioactive material of the present invention comprises a ceramic. Compositions such as disclosed in U.S. Patent No. 4,478,904; PCT/US94/13152; and Paul Ducheyne, "Bioglass Coatings and Bioglass Compositions," *J. Biomedical*  
10 *Materials Research*, 19:273-291, 1985 (all references hereby incorporated by reference) are also contemplated. In either glass or ceramic forms, however, the material is preferably porous, and the porosity is between about 20-30% and the pore size range is about 75-200  $\mu\text{m}$ .

15 The present invention therefore discloses methods of forming a porous glass substrate comprising the steps of melting an admixture consisting essentially of  $\text{SiO}_2$ ,  $\text{Na}_2\text{O}$ ,  $\text{CaO}$  and  $\text{P}_2\text{O}_5$  and then quenching the melted admixture to create a glass frit. A glass powder is formed from the glass  
20 frit that preferably has a particle size between about 40 to 70  $\mu\text{m}$ . A porous glass substrate is then formed from this powder by a foaming process. An important aspect of the present invention is that the resulting porous glass exhibits substantially no crystallization. Crystallization can  
25 occasionally be acceptable, but its main drawbacks are the nonuniform corrosion rates of the surface, and therefore the spatial variation of the reaction layers. Furthermore, the corrosion reactions are considerably slower, thereby necessitating long conditioning and incubation times.  
30 Alternatively, the porous glass substrate can be formed by creating a slurry of glass powder and a binder such as polyvinyl alcohol and pouring the slurry into a mold, drying the slurry and sintering it to produce a porous glass substrate. Finally, the porous glass substrate can be formed  
35 by first quenching the admixture so that a glass is formed. This glass is transformed into a glass ceramic having more than one crystalline phase and then preferentially dissolved



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to result in a porous material. The preferential dissolution may be accomplished by, for example, adding a solvent such as an acid or a base.

The present invention thus discloses the formation of  
5 a porous, preferably non-crystalline, bioactive glass. It is anticipated, though, that any known bioactive glass or ceramic can suitably be transformed to become a template for *in vitro* synthesis of bone tissue. Inoculation times will probably differ substantially. The glass material disclosed  
10 herein can be formed into a prosthetic article such as a disk, a sleeve, a rod or any other form of substrate. Alternatively, the glass formed herein can be provided in the form of particles.

In order to enhance bioactivity and bone formation  
15 using the glass of the present invention, the glass must be treated to prepare its surface for cell attachment and to control pH prior to its inoculation with cells. Therefore, the present invention also discloses methods of forming bone tissue comprising the steps of providing a porous bioactive  
20 template consisting of the glass material described above, immersing the template in a buffer solution and immersing the template in a tissue culture medium to produce a surface which significantly enhances bone cell activity when cells are inoculated on this surface. Finally, the template is  
25 inoculated with cells and bone tissue is permitted to form thereon. Most preferably, the buffer solution is buffered at a pH of about 6.8 and the tissue culture medium has a pH not exceeding about 7.6. The cells inoculated on the template may be chosen from any preparation providing osteoprogenitor  
30 cells, bone marrow cell preparations, cells of osteoblastic phenotypic potential or of osteoblastic phenotype. Inoculation may also proceed with fibroblasts and chondrocytes.

The bioactive materials can also be treated to achieve  
35 an enhanced conformational state of subsequently adsorbed adhesion molecules and greater attachment strength of anchorage-dependent cells in general. As is discussed in

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more detail below, greater attachment strength signifies better cellular response, *in vitro* and *in vivo*. The treatment involves immersion in a first aqueous solution containing a concentration of ions typical of interstitial fluid followed by at least a second immersion in an aqueous solution containing at least one adhesion molecule.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a photomicrograph of a cross-section of a substrate made in accordance with the present invention illustrating the formation of bone-like sheets.

Figure 2 is a photomicrograph of the surface of bioactive glass made in accordance with the present invention showing globular accretions connected by collagen fibrils.

Figure 3 depicts fibronectin (Fn) adsorption as a function of coating solution concentration.

Figure 4 depicts critical shear stress as a function of adsorbed Fn.

Figure 5 depicts a detachment profile for surfaces coated by immersion in a solution containing 0.1 micrograms per ml Fn.

#### DETAILED DESCRIPTION OF THE INVENTION

In the field of *in vivo* bone growth, glasses are known to produce a beneficial effect beyond what calcium phosphate ceramics produce. See e.g. Schepers et al. "Bioactive glass particulate material as a filler for bone lesions," J. Oral Rehabilitation, 8, 435-452 (1991). However, this effect *in vivo* has not been reproduced *in vitro*. The present invention discloses a new physical form of glass along with an additional new surface treatment that is effective *in vitro*.

Although porosity of a substrate is not essential, it is advantageous in view of the large specific surface area in potential contact with cells. The growth of tissue through the substrate leads to a fast formation of tissue needed for reimplantation and the artificial material degrades faster. One aspect of the present invention therefore provides

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methods for making bioactive glass porous. It will be realized that the methods as proposed in the prior art would have produced a crystallized glass, and crystallization must be avoided since it interferes with bioactivity reactions.

5 In other words, the dissolution and ion exchange reactions leading to a calcium-phosphate surface, possibly a carbonated calcium-deficient hydroxyapatite very similar to the mineral phase of bone, are slowed down substantially with crystals being present in the glass. In the limit, it is possible

10 that the necessary calcium-phosphorus rich layer would be formed so slowly that it is of minimal or no consequence to the cellular attachment reaction. If cellular attachment is not achieved, it can be assumed that cellular function will be hampered, if not impeded. Thus, when specifically

15 focusing on the *secretory* function, that is the extracellular matrix formation or the bone tissue-like synthesis, it would be severely reduced and, therefore, no true utility would be achieved. It should be noted at the outset that the present invention does not rely on cell biology. Many cells that

20 have the potential to be upregulated to cells of the osteoblastic phenotype can be used.

The applications of the *in vitro* synthesis templates disclosed herein may be described by analyzing the advantages and disadvantages of granules of bioactive glass. See

25 Schepers et al. "Bioactive glass particulate material as a filler for bone lesions," J. Oral Rehabilitation, 8, 435-452 (1991). One of the great advantages of using the glass in the form of granules is that they are presently ready for clinical use, and the *in vitro* synthesis templates are not.

30 Since both are glass based products, the acceptance in the market of granules may greatly help the future introduction of the templates.

It is worthwhile to note that the template disclosed herein can be made in porous particle form. This enables the

35 templates to be used for filling the many different forms of defects one encounters in surgery. However doing so forgoes one of the significant attributes of the templates, namely

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that they can come as large rigid structures. This is of great significance in any situation of either trauma or pathology, where bone loss is substantial and where the repair and reconstruction must be attempted with solid blocks to give the necessary rigidity to the structure after surgery. In such situations it is a limitation of granules or particles that they form a paste, since they can be squeezed from the site of delivery.

A second major advantage of *in vitro* synthesized bone tissue formation on templates made in accordance with the present invention is that they can be implanted into sites with otherwise limited growth potential. Cells which have been seeded onto the template in accordance with the methods of the present invention have been made active before implantation. This observation opens up enormous perspectives. Our Western populations are growing ever older and it is in this older age group that osteoporosis is prevalent. Osteoporosis, characterized by greater bone resorption than formation, afflicts more than fifty percent of the population beyond fifty-five years of age. It leads to the gradual compression of the spine and the typical hunch back. Osteoporosis is also the basis of a large percentage of hip fractures, with their considerable morbidity, and even significant secondary mortality. In short, the seeded glass surfaces with *in vitro* grown bone tissue disclosed herein can be inserted in all areas of the body that exhibit an increased risk of fracture and a decreased or even extinct potential for bone tissue formation. The network of porous, bioactive glass of the present invention can be made such that it resembles the porous bone network in the vertebrae of the spine, or the neck of the hip bone.

The cells seeded on to the synthetic substrate disclosed herein are most preferably cells from the patient. In experiments, osteoblasts, i.e., the cell which secretes the bone tissue material, have been used. However, as well known to those of ordinary skill osteoblasts are merely the end stage cell of cells of osteoblastic lineage. It is also

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possible, to use cells which are precursor cells to osteoblasts, that are typically present in the bone marrow. A simple extraction, followed by an appropriate separation and conditioning, yields cells that can be seeded. Intra-  
5 family donation of these cells are planned for those cases where either the aging of the patient to be treated has progressed to the point where he has only a limited cell activity anymore, or where the clinical circumstances are such that cell biopsies cannot be obtained.

10 A third important advantage of the porous bioactive glass templates disclosed herein is that they can be implanted in sites where there is an immediate need for bone. Once the templates have been seeded, results indicate that bone tissue has already formed extensively *in vitro* after one  
15 week.

In summary, the present templates will not fully replace the nonporous granules discussed in the Schepers reference noted above, rather they will broaden the indications for which these granules were used before in a  
20 rather restrictive way. Furthermore, they will create totally new therapies, such as the ones in osteoporotic pathology as suggested above.

Those of skill in the art will recognize that these porous templates will also become used in primary joint  
25 replacements. A porous coated prosthesis will be surrounded by a sleeve of the porous template material made in accordance with the present invention, and seeded with cells. After about one to two weeks, it will be inserted into the patient. At that time, a very expeditious incorporation of  
30 the device into the native femoral bone will occur.

Another new and useful area to which the present invention can be applied is the delivery of drugs or biological molecules to a patient. By adding a drug or biological molecules to the substrate, other therapeutic  
35 effects in addition to bone replacement can be achieved.

In addition to the foregoing, the present invention also contemplates treating bioactive glasses and ceramics to

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facilitate the attachment and activity of anchorage-dependent cells in general, and not just bone cells. As their name implies, anchorage-dependent cells must be anchored to a substrate to function. Some examples of anchorage-dependent cells include, but are not limited to, osteogenic cells, fibroblasts, neural cells, endothelial, epithelial cells, keratinocytes, chondrocytes, myocytes, hepatocytes, cells from joint ligaments, and cells from the nucleus pulposus. For such cells, the conditions of attachment of the cells to a particular substrate greatly influences their subsequent function.

Cell attachment to substrates is one of the events leading to tissue formation and may be influenced by the surface composition and structure of the synthetic or biological substrate, protein adsorption, physiological fluid forces, and the chemical environment affected by the surface reactions. The influences upon cell attachment has implications in both *in vitro* and *in vivo* settings.

Cellular attachment to extracellular matrices is usually mediated by integrins, a family of calcium-dependent surface receptors. R.O. Hynes. Integrins: versatility, modulation, and signaling in cell adhesion. [Review]. Cell 69:11-25, 1992. R.O. Hynes. Fibronectins, Springer-Verlag: New York, 1990 (both references incorporated herein by reference). These proteins bind to several extracellular matrix (adhesion) molecules, including fibronectin, vitronectin, various collagen types, including collagen type I, osteopontin, bone sialoprotein thrombospondin, and fibrinogen. Many of these macromolecules are adsorbed from serum and other biological fluids onto implant surfaces, thereby mediating cellular attachment and influencing physiological behavior.

Various studies, consisting mainly of morphological and biochemical analyses, have examined the behavior of different cell types, including fibroblasts and osteoblasts, cultured on hydroxyapatite and bioactive glasses. Several of these studies researched the effect of fibronectin on the

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adhesion of fibroblasts to bioactive glass. Seitz et al.,  
Effect of fibronectin on the adhesion of an established cell  
line to a surface reactive biomaterial. J. Biomed. Mat. Res.  
16:195-207, 1982; and Cannas et al., Bioimplant surfaces:  
5 Binding of fibronectin and fibroblast adhesion. J. Orthop.  
Res. 6:58-62, 1988. Others have reported on hydroxyapatite  
composites which include a synthetic fragment of the cell  
attachment site of collagen used as bone graft material.  
Parsons et al., "In Vivo Evaluation of Anorganic Bovine HA  
10 with Adsorbed Bioactive Synthetic Peptides," The 20th Annual  
Meeting of the Society for Biomaterials, April 5-9, 1994,  
Boston, MA, p. 125. Although some of these studies suggest  
that protein adsorption may be important for cellular  
adhesion to these materials, the phenomenon and mechanism of  
15 cell attachment had not been well characterized. Therefore,  
it could not be optimized.

Further, others have reported that the conformation of  
the bound fibronectin is important for subsequent function.  
Grinnell et al., "Adsorption characteristics of plasma  
20 fibronectin in relationship to biological activity," J.  
Biomedical Materials Research, 15:363-381, 1981, hereby  
incorporated by reference. The work of Grinnell et al.  
focused upon adsorption to wettable and non-wettable culture  
plates, i.e., polystyrene. Considering the important role  
25 attachment molecules such as fibronectin play in cell  
functioning, and in the overall cascade of cell-signalling  
events, it is clear that the conformation of the adsorbed  
molecules is critical. The treatments disclosed below afford  
significant improvements by enhancing conformational state of  
30 the adsorbed adhesion molecules.

The effects of applied detachment force, fibronectin  
adsorption, and surface reaction treatment on the initial  
attachment of an anchorage-dependent cell to bioactive glass  
were specifically examined and are disclosed below. As the  
35 results show, the strength of attachment to the bioactive  
surfaces was over two-times greater when the glasses were  
subjected to a first treatment by immersion in aqueous

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solutions containing a concentration of ions typical of interstitial fluid. Similar results are expected with other adhesion molecules. The amount of fibronectin adsorbed, as well as the number of cells deposited onto the surface before application of any detachment force, did not differ significantly according to the surface or treatment. Accordingly, the over two-fold differences in strength of attachment observed with the bioactive material subjected to the first treatment, as compared to the bioactive material not subjected to the first treatment, were unexpected. Since there were no differences in the amount of fibronectin adsorbed, it was thus concluded that the strength of attachment is a result of the enhanced conformational state of adhesion molecules when adsorbed to surfaces subjected to the first treatment described hereinabove. The greater attachment strength, in turn, signifies improved cell function and, thus, more expeditious tissue growth both *in vivo* and *in vitro*.

The concentration of adhesion molecules is selected such that the desired level of attachment is achieved; saturation of the surface is not required. If more than one adhesion molecule is to be used, concentrations can be adjusted accordingly. Alternatively, the invention contemplates a three-step immersion, the second and third immersions being performed in aqueous solutions of different adhesion molecules, performed sequentially. Of course, in the instance of three immersions, the concentration of the adhesion molecule in the second immersion is preferably not surface-saturating.

Although the duration of immersion in the solutions containing the adhesion molecules is dependent upon concentration, incubation times of at least 20 seconds are contemplated, a preferred incubation time being 20-30 minutes. The immersion solution volume is selected so that the pH of the solutions is and remains at a value tolerated by physiological systems. Preferably, the pH variation during treatment is  $7.4 \pm 0.2$ .



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As can be seen from the foregoing, not only can a treatment according to the invention with adhesion molecules, or combinations thereof, promote the expeditious growth of bone tissue *in vitro*, but it can also promote the expeditious growth of other tissues, *in vivo* or *in vitro*.

As used herein, the term "contact" in reference to anchorage-dependent cells includes both *in vitro* and *in vivo* events. The term includes, but is not limited to inoculation through immersion and implantation.

The term "implant" refers to a material for treating defects in sites where tissue is made by anchorage-dependent cells. The defects can be a result of injury, disease, surgery, wear and tear, or old age. For example, without meaning to be limited thereby, implants for the regeneration of bone tissue, blood vessels, and nerves are contemplated.

The implant preferably comprises a silica-based glass further comprising calcium. The implant can be made either by melt-derived or sol-gel technology, and can be in the form of granules, discs, blocks, monoliths, fibers, or sheets. The implant can comprise the substrate of bioactive material as disclosed herein, or simply be coated with the substrate. In a further embodiment, the implant comprises anchorage-dependent cells inoculated thereon prior to implantation. The term also includes prosthetic devices which, according to the invention, can have a coating, or partial covering, of said substrates.

#### Example 1

##### GLASS PREPARATION

Bioactive glass with a nominal composition (% by weight) 45%  $\text{SiO}_2$ , 24.5%  $\text{Na}_2\text{O}$ , 24.5%  $\text{CaO}$  and 6%  $\text{P}_2\text{O}_5$  represents a most preferred embodiment and is prepared from chemically pure reagent grade  $\text{Na}_2\text{CO}_3$ ,  $\text{CaCO}_3$ ,  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$  and  $\text{SiO}_2$ . However, the following ranges of these constituents are useful to form the material of the present invention: 40-60%  $\text{SiO}_2$ ; 5-30%  $\text{Na}_2\text{O}$ ; 10-35%  $\text{CaO}$  and 0-12%  $\text{P}_2\text{O}_5$ . The prorated amounts of the reagents are admixed and melted in a crucible

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at about 1350 C. The homogeneity of the melt is assured by swirling the melt-containing crucible as required, but most preferably at least three times. The melt is then poured into deionized water. The glass frit obtained by this method is then dried and used for the synthesis of porous glass. For the synthesis of porous glass, the glass frit is crushed and ground into a powder with a particle size in the range of about 40-70  $\mu\text{m}$ . This powder is admixed with 2-3%  $\text{CaCO}_3$ , which is used as foaming agent. The mixture is hot pressed in vacuum at about 50 MPa and 460 C for about two hours. The resulting pore size range is about 70-200  $\mu\text{m}$ . A larger pore size range, e.g., about 200-500  $\mu\text{m}$ , is obtained by using either  $\text{CaCO}_3$  powder of a larger particle size, or by using  $\text{NaHCO}_3$  as the foaming agent. The average porosity and pore size is determined by weight measurements and stereometry, respectively. An X-ray diffraction analysis of porous bioglass prepared in accordance with the present invention shows complete vitrification with no sign of crystallization after the foaming process.

\* \* \*

In order to optimize the synthesis of glass in accordance with the present invention, several foaming agents ( $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ ,  $\text{NH}_4\text{H}_2\text{PO}_4$  and marble) with different concentrations (1%, 2%, 3%, 6% and 10%) and a number of sintering and foaming treatments (at 450, 580, 475 and 550 C) for different periods of time (1, 2, 3, and 5 hours) were used. Double stage heat treatment (450 C for 1 hr; then heated at either 475 C, 550 C, or 580 C for 1 hr) was also used. Glass powder of different grain sizes, e.g., about 110  $\mu\text{m}$  and between about 70 - 40  $\mu\text{m}$  were used and the applied pressure was also taken as a variable and pressures of about 100, 70, and 40 MPa were tried. Those foaming agents and processing conditions that did not measurably initiate a glass crystallization reaction are preferred. Any crystallization renders glass more corrosion resistant or produces a nonuniform corrosion. This must be avoided as it

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adversely affects the beneficial formation of the surface reaction layers.

The best result was obtained by admixing the glass powder of grain size between about 40 and 70  $\mu\text{m}$  with about 2.3%  $\text{CaCO}_3$  that is pressed in a graphite die at a pressure of about 50 MPa. The pressed admixture is then heated at about 460 C for about two hours in a vacuum press. Using high pressure, e.g., about 100 MPa and heat treatment at about 580 C produces non-porous glass-ceramic material, so these values for these variables are not preferred. Moreover, it was found that using low pressure, e.g., about 40 MPa, produces a fragile material that can not be handled. Heat treatment of long duration will initiate crystallization of the glass, especially in the presence of a foaming agent, so the shortest heat treatment duration represents a most preferred embodiment.

Alternative processes useful with the present invention to produce porous glass, glass-ceramic, or ceramic are:

#### (1) Foaming and Sintering

By making an aqueous ceramic slurry with a binder (e.g., polyvinyl alcohol), and then mixing the slurry with a foaming agent (e.g.,  $\text{H}_2\text{O}_2$  or  $\text{CaCO}_3$ ) pouring the mixture into a mold, allowing it to dry, and then sintering it at high temperature useful material may be produced. This method is less preferred because it can change the nature of the glass surface and hence limit the bioactivity.

#### (2) Ceramization and Leaching

By crystallizing the glass into more than one crystalline phase, and then treating the obtained glass ceramic with acid or base that preferentially dissolves one phase and leaves the others, porous material is created. The ceramization and leaching process represents a less preferred embodiment, however, because leaching of calcium phosphate-rich phase will decrease the bioactivity of the glass.

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### (3) Sol Gel Method

As well known in the art, controlling the heat treatment cycle of the ceramic or glass gel controls the pores and interpores of the material and thus a porous ceramic can be obtained. However, the control of pore size and porosity percent of bioactive glass prepared by this method is much more difficult to obtain in the desirable range than sintering and foaming process described above.

#### Example 2

#### 10      **CONDITIONING OF THE GLASS SURFACE AND CONTROL OF pH VARIATION**

Another important aspect of the present invention is the conditioning of the glass surface with concurrent pH control of the solution. Such conditioning is essential for two reasons: (1) if cells are added to unconditioned glass, the glass corrosion reaction which takes place would lead to pH values at the surface of the glass and in the bulk of the solution that would kill the cells; and (2) the treatment must make the surface ready for expedient cell attachment and extracellular matrix deposition. Before describing the best conditioning of the glass we first describe in a chronological fashion, some of the experiments performed.

Glass disks made in accordance with the techniques described above were soaked in tris buffer solution containing ions in a concentration similar to that found in interstitial fluid, and shaken on a rotary table at 37 C (pH 6.84) for 48 hours. The volume of this solution was one liter. No change in the pH value was measured during the time period of glass immersion. Electron dispersive X-ray analysis (EDXA) revealed that a calcium phosphate rich layer had formed on the surface of the bioactive glass.

In another experiment, the glass disks were either treated in modified tris buffer solution for 20 hours, or treated for 20 hours in tris buffer followed by 48 hours in a typical tissue culture medium (TCM), i.e. E-MEM (Eagles' minimal essential medium + 10% NBS + 2 mM L- glutamine) (treatment TB2-E-MEM), then inoculated with cells. Four or

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five disks were immersed. The solution was maintained at 37 C. A pronounced difference was observed in number of cells attached to the glass disks and secretion of extracellular matrix material. Comparison of the number of cells attached to these two differently treated glasses showed more cells adhered to the glass disks treated in tris buffer alone. Both sets of samples showed collagen fibrils and calcified nodules, yet, neither showed any bone-like tissue formation. The cells attached to the glass disks treated according to TB2-E-MEM produced more collagen fibrils in between the cells. Since cell attachment represents a preferred embodiment, in our subsequent experiments we now assumed that immersion in the modified tris solution was preferable. In this next set of experiments we then addressed the question whether, if we inoculated cells on these disks after treatment in modified tris for 20 hours, there would be a pH variation with the potential of killing of the cells. In these experiments we treated the disks for 20 hours in modified tris and subsequently immersed the disks in the same tissue culture medium as we would use for the inoculation and incubation of cells and we measured the pH variation. We did this first for one ratio of the tissue culture medium volume to glass weight. Thus, we treated glass disks in tris buffer at 37 C for 20 hours followed by immersion in a E-MEM at 37 C. The ratio of solution volume to glass weight was about 19 ml/g. The corrosion products of the glass were found to increase the pH of the TCM solution from 7.6 to 7.9 after 24 hours, and from 7.6 to 8.0 after 48 hours. This increase in pH may have an adverse effect on cells if they were inoculated in such solution, i.e., the cells would die.

A next series of experiments used different ratios of TCM volume to glass weight. Based on the results of the last experiment, we also increased the duration of immersion in tris from 20 to 48 hours. This was intended to minimize subsequent dissolution from the glass into the TCM, when cells would be present. As before, there was a two step immersion: immersion in modified tris buffer solution for 48

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hours, and then immersion in TCM for three days. The initial pH of the TCM is 7.6. The pH of the TCM was measured after 24, 48, and 72 hours. The TCM was then replaced by TCM + 3 mM B-glycerol phosphate, and the pH was also measured every 5 24 hours for 3 days. The minimum TCM volume/glass weight ratio that did not change the pH value of the TCM was 90 ml/g, yet there may still be a pH gradient between the glass surface and the bulk of the TCM. Furthermore, the pH value of the TCM inside the pores may be considerably higher than 10 the one of the TCM in the bulk of the solution in the dish. We found that when the pH of the bulk TCM raised to about 7.85, for example, due to a low ratio of TCM volume to glass weight, the activity of the cells that were seeded on the preconditioned glass disks was greatly inhibited. Scanning 15 election microscope (SEM) analysis of these cultures showed very limited cell processes and the alkaline phosphatase activity was minimal, as followed from the faint pink color seen when samples were stained with fast red staining.

Based on the these last results, we performed yet 20 another experiment in which we now added a strong biological buffer to the tissue culture medium: we added 20 mM Hepes. We added this buffer in order to stabilize the pH value inside the pores. Thus, the total set of conditions was: immersion for 48 hours in modified tris, immersion in E-MEM 25 complemented with 20 mM Hepes and a 90 ml/g ratio of TCM volume to glass weight. The second immersion, then, represents conditions for inoculation. Thus, when the pH in the pores and at the surface of the glass was stabilized at 7.6 during the period the cells were seeded on the glass, 30 abundant cell processes were possible and bone like tissue formed extensively after only one week in culture.

The glass disks are dried, and most preferably sterilized by ethylene oxide prior to inoculation with cells.

The effect of the immersion treatment being the 35 formation of calcium phosphate rich reaction layer is known. See Hench et al. "Bonding mechanisms at the interface of ceramic prosthetic materials," J. Biomed. Mater. Res. Symp.,

- 21 -

2:117-141 (1972). This reaction layer gradually matures into a poorly crystallized, defective calcium hydroxyapatite structure. See Kim et al. "Early stages of calcium phosphate layer formation in bioglass," J. Non-Cryst. Solids, 5 113:195-202 (1989). The immersion treatment of the present invention, however, differs in a fundamental way from the method used by Hench et al., referenced above. The intent of the prior art was to establish kinetics of the several ion exchange, dissolution, condensation, precipitation and 10 transformation reactions at the glass surface. For these reasons, a tris-buffered solution which does not contain any of the typical components of interstitial bone fluid was used. In contradistinction, the present invention achieves a glass surface resembling the mineral phase of bone upon which 15 biomolecules are absorbed as soon as the glass is brought into contact with a solution containing biological molecules. This can be achieved by either of two ways: i) prior to inoculation but after immersion in modified tris buffer, the disks are immersed in TCM or any other suitable solution 20 containing biological molecules normally occurring in bone tissue; or ii) the disks are immersed in the cell suspension containing biological molecules. Before the cells adhere, the biological molecules attach to the glass surface and incorporate into the continuously developing glass surface 25 reaction layer. The result is that the developing calcium phosphate layer is coated and intimately mixed with organic molecules.

### Example 3

#### 30 CHARACTERIZATION OF *IN VITRO* SYNTHESIZED BONE TISSUE

After the above described modified tris buffer pretreatment, the 10 mm diameter disks are placed in 60 mm diameter petri dishes or other suitable container, wetted with TCM and inoculated with about one million neonatal rat 35 calvaria osteoblasts from a suspension containing 1.2 million cells/ml. The cell suspension used herein was obtained by an enzymatic digestion method using 0.2% collagenase. To allow

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for cell attachment, the petri dishes were kept in the incubator for about one hour prior to flooding them with TCM. Two days later, the TCM was exchanged by TCM supplemented with 3 mM  $\beta$ -Glycerol phosphate. This exchange was repeated  
5 at day four. The dishes were sacrificed at day seven. Morphological analysis by using scanning electron microscopy (SEM), energy dispersive x-ray microanalysis (EDXA) for calcium, phosphorus and sulfur, qualitative and quantitative determination of alkaline phosphatase concentration in  
10 solution, and DNA, collagen I and osteocalcin synthesis reveal the properties of the tissue that had formed.

The porous specimens were found to be totally invaded by the cells and the extracellular matrix they elaborated, as seen in FIG. 1. FIG. 1 shows a scanning electron micrograph  
15 (x100) of a cross-section through the porous bioactive glass after 1 week of incubation. Bone-like sheets were formed throughout the whole thickness of the porous glass sample. FIG. 2 shows another scanning electron micrograph (x3500) of the bioactive glass surface. The photograph was made at a  
20 time the parameters of our invention were not yet optimized. It shows some of the events prior to the extensive bone tissue formation: globular accretions in direct contact with the glass surface are visible. Collagen fibrils interdigitate with these globular nodules. The calcified  
25 cement-like layer is not yet covered by the bone-like sheets and is thus still visible.

The qualitative assessment of alkaline phosphatase activity reveals that the highest activity is with the cells on the bioactive glass disks; there is only a faint activity  
30 on the bottom of the petri dishes around the glass disk.

The quantitative determination of the alkaline phosphatase activity expresses an average per dish. Using the method of quantifying released para-nitrophenol (PNP) set forth in Lowry et al. "The quantitative histochemistry of the  
35 brain: II. Enzyme measurements," J. Biol Chem., 207:19-37 (1954), a rate of 0.62 nmol pNP/min/ $\mu$ g protein was found. As for  $\mu$ g DNA, a typical value (2.01  $\mu$ g/ml) for osteoblastic



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phenotypic expression was indicated. The extensive morphologic observation of the various samples suggests the following hypothetical sequence of events: in a first step the foci of calcification form: small globules, 1-2  $\mu\text{m}$  in diameter, appear on the glass surface. Subsequently, collagen fibrils are produced and attach to the calcified nodules.

It should be noted that collagen I synthesis has been confirmed. From thereon, the synthesis of both calcified substance and intertwined fibrils continues, thereby leading to the gradual formation of bundles and eventually the coalescence into sheets of bone-like material. It is important to note that the surface of these sheets is smooth and free of calcified globules. Such observations are intriguing as they fit the theory that these calcified globules serve to bridge the collagen fibrils and thereby create the continuous bone-like sheets. It is another intriguing observation that without the initial elaboration of these nodules on the glass surface, the cells do not produce collagen fibrils. Osteocalcin radioimmunoassay (using goat anti-rat osteocalcin, donkey anti-goat second antibody and I-125 rat osteocalcin) showed a considerable concentration (about 8 ng/ml) of osteocalcin. Osteocalcin was detected only on those dishes containing glass and osteoblasts. Control dishes with osteoblasts but without glass disks never showed appreciable concentration of osteocalcin.

The calcification of elaborated material was not the result of a physio-chemical phenomenon of precipitation from a solution supersaturated in calcium and phosphorus, this in itself resulting from glass dissolution. Control experiments with glass without inoculation of cells, or cell culture experiments without porous bioactive glass did not produce a measurable calcification.

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## Example 4

## IMPROVED CELLULAR ATTACHMENT STRENGTH

Bioactive glass (45% SiO<sub>2</sub>, 24.5% CaO, 24.5% Na<sub>2</sub>O, 6% P<sub>2</sub>O<sub>5</sub>) disks (15 mm dia.) were prepared using a melt-derived technique. The disks were first treated in an aqueous solution containing a concentration of ions typical of interstitial fluid (80 mM tris buffer + 142 mM Na<sup>+</sup>, 5.0 mM K<sup>+</sup>, 2.5 mM Ca<sup>+2</sup>, 1.5 mM Mg<sup>+2</sup>, 135 mM Cl<sup>-</sup>, 27 mM HCO<sub>3</sub><sup>-</sup>, and 1.0 mM HPO<sub>4</sub><sup>-2</sup>, pH=7.4) at 37°C for 0, 1, or 7 days to produce three distinct reaction layers: (i) unreacted glass (BG0); (ii) amorphous calcium-phosphate (BG1d); and (iii) carbonated hydroxyapatite (BG7d). The reaction stages were verified by FTIR. Borosilicate glass (CG) was used as control.

Human plasma fibronectin (Fn) (Sigma Chemical Company) was diluted in the above aqueous solution and radiolabeled with <sup>125</sup>I using the Bolton-Hunter reaction. The concentration of Fn ranged from 0 to 1.0 micrograms/ml. The substrates were incubated at 37°C in <sup>125</sup>I-Fn solutions for 30 minutes, then in 1% bovine serum albumin (BSA) for 30 minutes. The adsorbed Fn was quantified using a gamma counter.

The amount of adsorbed Fn (Γ) increased with increasing coating solution concentration (Figure 3). For the concentrations examined, there were no differences in the amount of adsorbed Fn for the different surfaces.

The strength of cellular attachment was then tested using the Cell Detachment Apparatus (CDA). The CDA was developed in our laboratories and is a spinning disk device that applies a linearly varying range of shear stresses to attached cells while maintaining uniform solute concentrations at the surface. Electrochemical measurements using the ferric/ferrocyanide redox reaction on a spinning platinum electrode demonstrated that the flow field for the CDA approximates that of an infinite spinning disk. For an infinite disk in laminar flow, the shear stress at the surface varies linearly with radial distance while the surface mass transport rate is uniform (Levich, F.G.,

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Physicochemical Hydrodynamics, Prentice Hall, Englewood Cliffs, N.J., 1962, hereby incorporated by reference).

The substrates were incubated at 37°C in Fn solutions for 30 minutes as described above, and then in 1% BSA solution for 30 minutes to block non-specific adhesion. Rat osteosarcoma (ROS 17/2.8) cells in culture were detached from tissue culture flasks using trypsin+EDTA+N-tosyl-L-phenylalanine chloromethylketone (TPCK) and suspended in adhesion buffer (Hank's balanced salt solution+10 mM HEPES+2 mM glucose)+N $\alpha$ -tosyl-L-lysine chloromethylketone (TLCK). Cells were seeded onto the disks (340 cells/mm<sup>2</sup>) and allowed to attach for 15 min. The disks were spun in adhesion buffer for 10 minutes and adherent cells were fixed, permeabilized, stained with ethidium homodimer, and counted automatically at specific radial distances.

The fraction of adherent cells, normalized to the number of cells at the disk center, was plotted as a function of shear stress and fitted to a logistic equation to estimate the critical shear stress for 50% detachment ( $\tau_c$ ).  $\tau_c$  was used as a measure of attachment strength. The adherent fraction decreased sigmoidally with shear stress. Furthermore,  $\tau_c$  increased linearly with adsorbed Fn density ( $R^2 = 0.98$ , Figure 4, CG omitted for clarity). There was no difference among the  $\tau_c$  for disks coated with BSA only (data not shown). For Fn-treated substrates,  $\tau_c$  was significantly higher for reacted bioactive glasses (BG1d, BG7d) than for control (CG) and unreacted bioactive (BG0) glasses ( $p < 0.05$ , Figures 4 and 5). There were no differences between BG1d and BG7d, or between BG0 and CG.

The fraction of attached cells decreased sigmoidally with applied detachment force, as expected for a normally-distributed binomial variate. In addition, the linear increase in attachment strength with Fn surface density is consistent with theoretical models (Hammer et al., "A dynamic model for receptor mediated cell adhesion to surfaces," *Biophys. J.* 52:475-87, 1987). The differences in adhesion strength among the surfaces indicate that the

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conformational states of adsorbed attachment molecules influence cell attachment. Surface treatment of bioactive glass enhanced Fn-mediated cell attachment. Surface treatment did not affect the amount of adsorbed Fn, suggesting that the enhancement in adhesion resulted from Fn conformational changes.

Although certain embodiments and applications of the present invention have been set forth with particularity, review of the foregoing specification will lead those of ordinary skill to realize that the present invention is not so limited. Numerous variations, modifications and application to other indications will immediately become apparent that do not depart from the spirit of the invention. For this reason, reference should be made to the appended claims in order to ascertain the true scope of the present invention.

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What is claimed is:

1. A bioactive glass or ceramic material having been treated prior to contact with anchorage-dependent cells to achieve greater cellular attachment strength.

5        2. The bioactive material of claim 1 wherein said material is immersed in a first aqueous solution containing ions in a concentration typical of interstitial fluid followed by immersion in a second aqueous solution containing at least one cell adhesion molecule.

10        3. The bioactive material of claim 2 wherein said at least one cell adhesion molecule is selected from the group consisting of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and  
15 fibrinogen, and combinations thereof.

      4. The bioactive material of claim 1 wherein said material is immersed in a first aqueous solution containing ions typical of interstitial fluid followed by immersion in a second aqueous solution containing a first cell adhesion  
20 molecule at a non-saturating concentration, followed by immersion in a third aqueous solution containing a second cell adhesion molecule.

      5. The bioactive material of claim 1 wherein said material is immersed in a first aqueous solution containing  
25 ions typical of interstitial fluid followed by immersion in a second aqueous solution containing a first cell adhesion molecule mixture at non-saturating concentration, followed by immersion in a third aqueous solution containing a second cell adhesion molecule mixture.

30

      6. The bioactive material of claim 1, wherein the glass consists essentially of:  $\text{SiO}_2$ ;  $\text{CaO}$ ;  $\text{Na}_2\text{O}$ ; and  $\text{P}_2\text{O}_5$ .

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7. The bioactive material of claim 6 having the following composition: 45% by weight  $\text{SiO}_2$ ; 24.5% by weight  $\text{CaO}$ ; 24.5% by weight  $\text{Na}_2\text{O}$ ; and 6% by weight  $\text{P}_2\text{O}_5$ .

8. An implant comprising a substrate of bioactive  
5 glass or ceramic treated to achieve greater anchorage-dependent cell attachment.

9. The implant of claim 8 wherein said substrate is immersed in a first aqueous solution containing ions typical  
10 of interstitial fluid followed by immersion in a second aqueous solution containing at least one cell adhesion molecule.

10. The implant of claim 9 wherein said at least one  
15 cell adhesion molecule is selected from the group consisting of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and fibrinogen, and combinations thereof.

11. The implant of claim 8 wherein said substrate is  
20 immersed in a first aqueous solution containing ions typical of interstitial fluid followed by immersion in a second aqueous solution containing a first cell adhesion molecule at a non-saturating concentration, followed by immersion in a third aqueous solution containing a second cell adhesion  
25 molecule.

12. The implant of claim 8 wherein said substrate is immersed in a first aqueous solution containing ions typical of interstitial fluid followed by immersion in a second  
30 aqueous solution containing a first cell adhesion molecule mixture at non-saturating concentration, followed by immersion in a third aqueous solution containing a second cell adhesion molecule mixture.

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13. The implant of claim 8 further comprising anchorage-dependent cells inoculated thereon prior to implantation.

14. The implant of claim 8 wherein the substrate is  
5 in the form of a sheet.

15. The implant of claim 8 wherein the substrate is in the form of granules.

16. The implant of claim 8 wherein the substrate is in the form of fibers.

10 17. The implant of claim 8 wherein the substrate is in the form of a coating on said implant.

18. A method of forming tissue made by anchorage-dependent cells *in vitro* comprising the steps of:

- 15 a) providing a substrate formed of a bioactive material;  
b) immersing the substrate in a first aqueous solution containing ions in a concentration typical for interstitial fluid;  
c) immersing the substrate in a second aqueous  
20 solution containing at least one cell adhesion molecule; and  
d) inoculating the substrate with cells in tissue culture medium.

19. The method of claim 18, wherein said aqueous solutions are at a pH of about 7.4.

25 20. The method of claim 18, wherein the tissue is bone tissue.

21. The method of claim 18 wherein said at least one cell adhesion molecule is selected from the group consisting of fibronectin, vitronectin, laminin, collagen, osteopontin,

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bone sialoprotein, thrombospondin, and fibrinogen, and combinations thereof.

22. A method of forming tissue made by anchorage-dependent cells *in vitro* comprising the steps of:

- 5 a) providing a substrate formed of a bioactive material;
- b) immersing the substrate in a first aqueous solution containing ions in a concentration typical for interstitial fluid;
- 10 c) immersing the substrate in a second aqueous solution containing a first cell adhesion molecule at a non-saturating concentration;
- d) immersing the substrate in a third aqueous solution containing a second cell adhesion molecule; and
- 15 e) inoculating the substrate with cells in tissue culture medium.

23. The method of claim 22 wherein said aqueous solutions are at a pH of about 7.4.

24. The method of claim 22 wherein the tissue is bone  
20 tissue.

25. The method of claim 22 wherein said first and second cell adhesion molecules are selected from the group consisting of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and  
25 fibrinogen, and combinations thereof.

26. A method of forming tissue made by anchorage-dependent cells *in vitro* comprising the steps of:

- a) providing a substrate formed of a bioactive material;
- 30 b) immersing the substrate in a first aqueous solution containing ions in a concentration typical for interstitial fluid;



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c) immersing the substrate in a second aqueous solution containing a first cell adhesion molecule mixture at non-saturating concentration;

d) immersing the substrate in a third aqueous  
5 solution containing a second cell adhesion molecule mixture;  
and

e) inoculating the substrate with cells in tissue culture medium.

27. The method of claim 26 wherein said aqueous  
10 solutions are at a pH of about 7.4.

28. The method of claim 26 wherein the tissue is bone tissue.

29. The method of claim 26 wherein said first and second cell adhesion molecule mixtures contain cell adhesion  
15 molecules selected from the group consisting of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and fibrinogen, and combinations thereof.

30. A method of preparing a substrate for attachment  
20 of anchorage-dependent cells comprising the steps of:

a) providing a substrate formed of a bioactive material;

b) immersing the substrate in a first aqueous solution containing ions in a concentration typical for interstitial  
25 fluid; and

c) immersing the substrate in a second aqueous solution containing at least one cell adhesion molecule.

31. The method of claim 30, wherein said aqueous solutions are at a pH of about 7.4.

30 32. The method of claim 30 wherein said at least one cell adhesion molecule is selected from the group consisting

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of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and fibrinogen, and combinations thereof.

33. A method of preparing a substrate for attachment  
5 of anchorage-dependent cells comprising the steps of:

a) providing a substrate formed of a bioactive material;

b) immersing the substrate in a first aqueous solution containing ions in a concentration typical for interstitial  
10 fluid;

c) immersing the substrate in a second aqueous solution containing a first cell adhesion molecule at a non-saturating concentration; and

d) immersing the substrate in a third aqueous  
15 solution containing a second cell adhesion molecule.

34. The method of claim 33, wherein said aqueous solutions are at a pH of about 7.4.

35. The method of claim 33 wherein said first and second cell adhesion molecules are selected from the group  
20 consisting of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and fibrinogen, and combinations thereof.

36. A method of preparing a substrate for attachment of anchorage-dependent cells comprising the steps of:

25 a) providing a substrate formed of a bioactive material;

b) immersing the substrate in a first aqueous solution containing ions in a concentration typical for interstitial fluid;

30 c) immersing the substrate in a second aqueous solution containing a first cell adhesion molecule mixture at non-saturating concentration; and

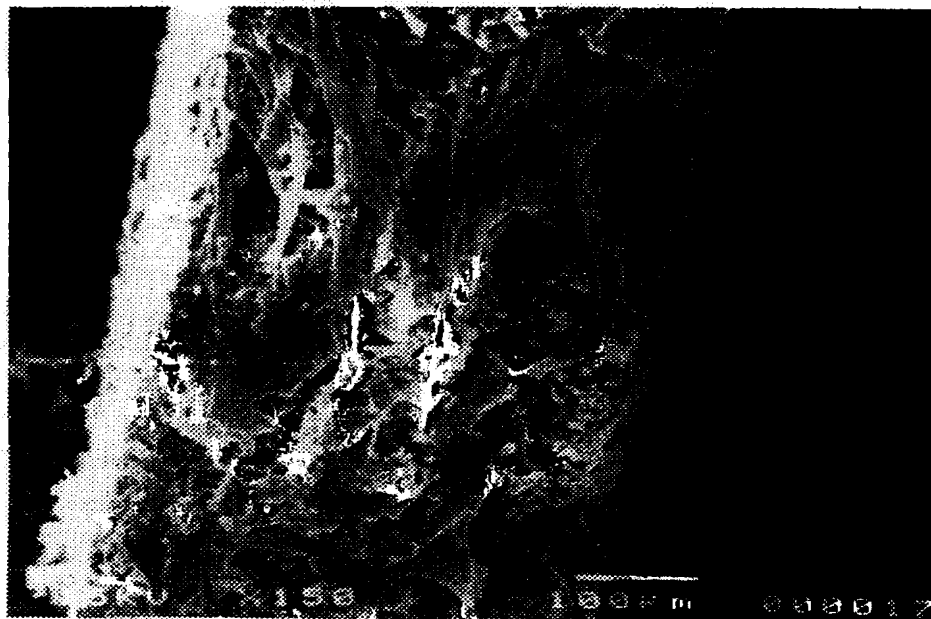
- 33 -

d) immersing the substrate in a third aqueous solution containing a second cell adhesion molecule mixture.

37. The method of claim 36, wherein said aqueous solutions are at a pH of about 7.4.

5        38. The method of claim 36 wherein said first and second cell adhesion molecule mixtures are selected from the group consisting of fibronectin, vitronectin, laminin, collagen, osteopontin, bone sialoprotein, thrombospondin, and fibrinogen, and combinations thereof.

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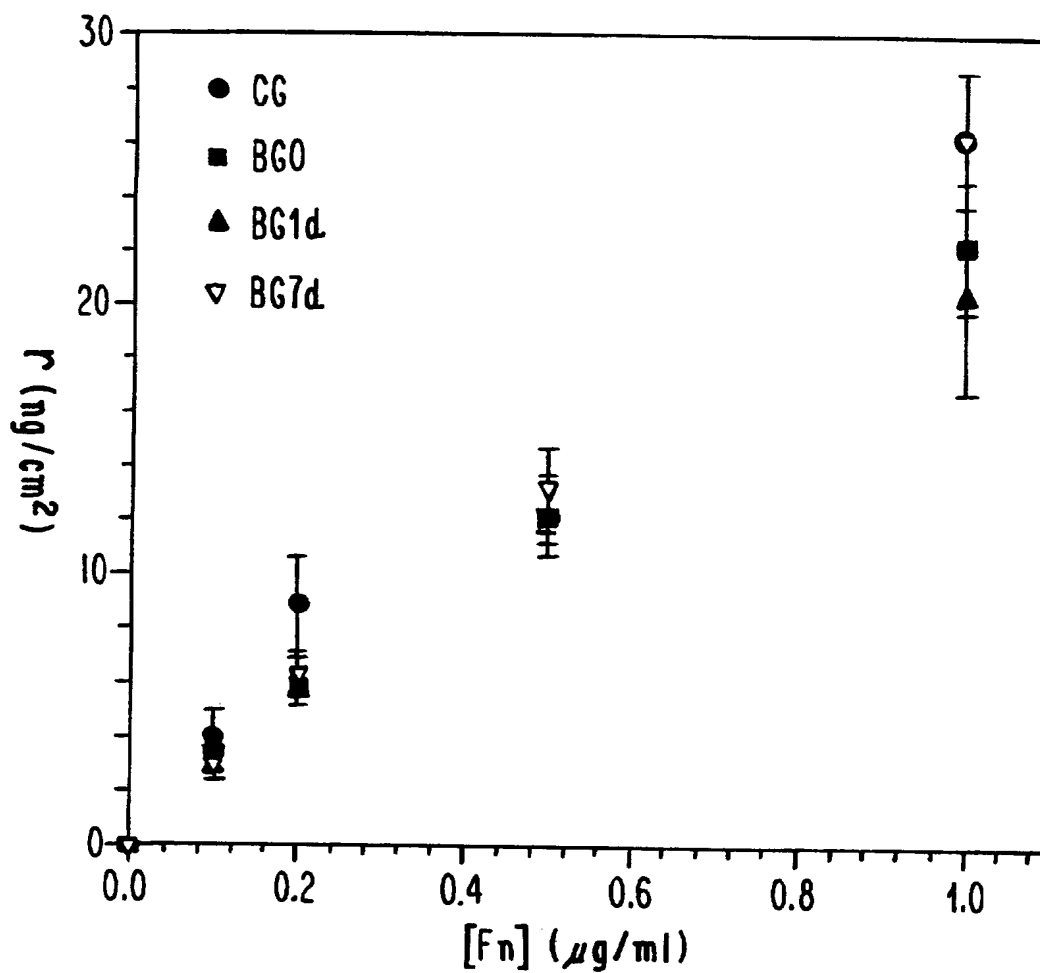
*FIG. 1*



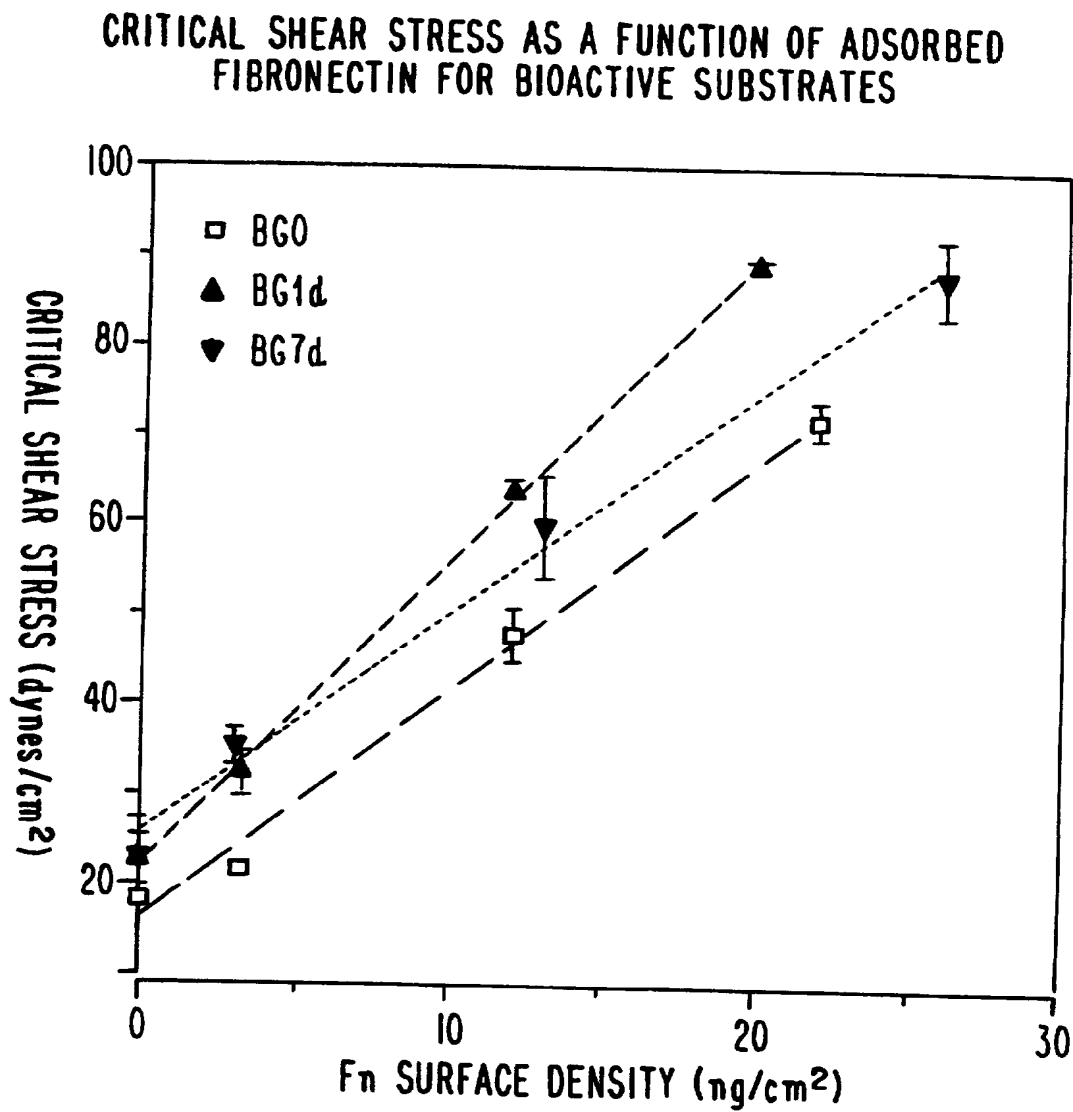
*FIG. 2*

SUBSTITUTE SHEET (RULE 26)

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FIBRONECTIN ADSORPTION ONTO BIOACTIVE MATERIALS AS A  
FUNCTION OF F<sub>n</sub> COATING SOLUTION CONCENTRATION (n=4)***Fig. 3***

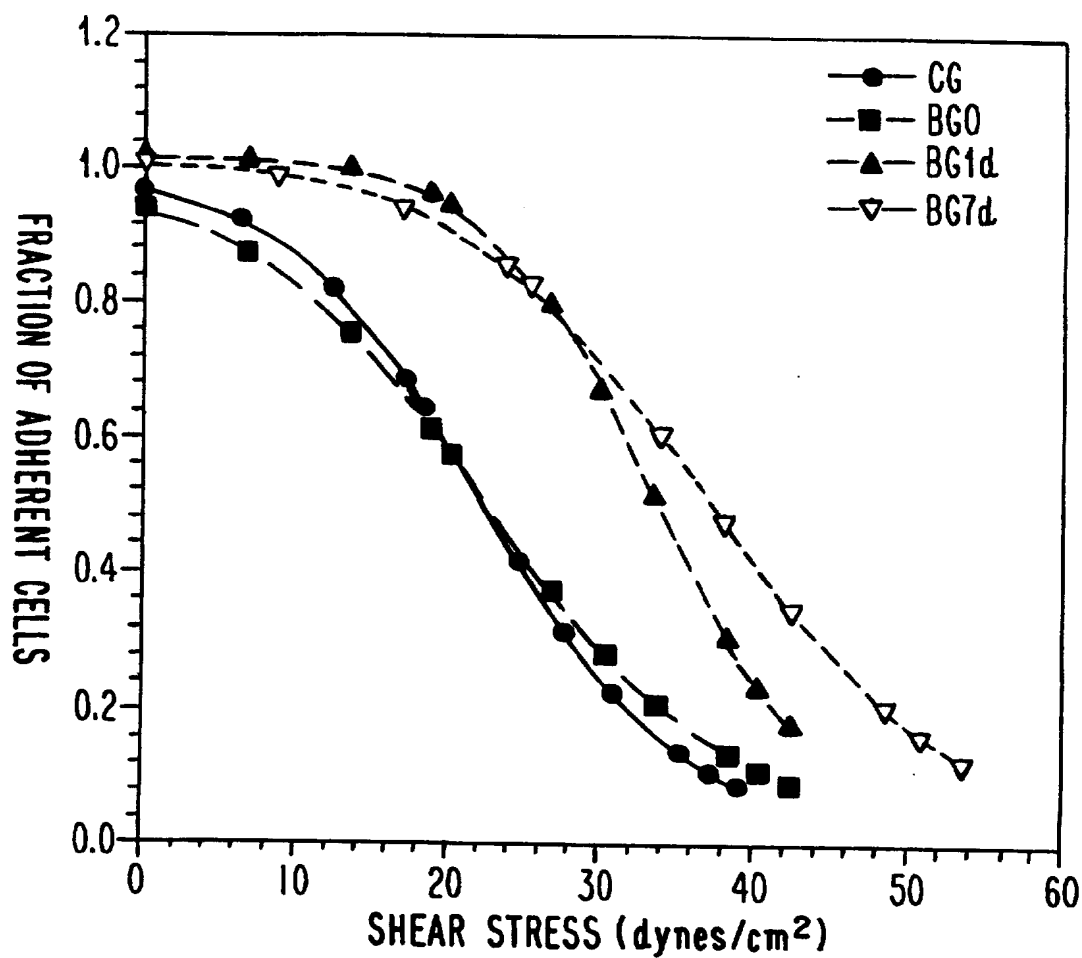
3/4



***Fig. 4***

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CELL DETACHMENT AS A FUNCTION OF APPLIED SHEAR  
STRESS FOR SUBSTRATES COATED WITH  $0.1\mu\text{g/ml}$



***Fig. 5***

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/04095

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 5/08, 5/00; A61F 2/28

US CL : 435/402, 1.1, 325, 378; 424/422; 623/16, 66; 530/356

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/402, 1.1, 325, 378; 424/422; 623/16, 66; 530/356

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, E	US 5,643,789 A (DUCHEYNE et al.) 01 July 1997, see whole document.	1-38
Y	El-Ghannam. 'Serum protein adsorption on bioactive ceramics and glasses and the effect on osteoblast adhesion' In: Society for Biomaterials, 21st Annual Conference, San Francisco, California, 18-22 March 1995, page 406.	1-38
Y	Garcia et al., 'A spinning disk device to examine receptor-mediated cell attachment to bioactive and surface-reactive materials', 2nd International Conference on Cellular Engineering, La Jolla, CA, August 1995, page 31.	1-38

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search 02 JULY 1997	Date of mailing of the international search report 24 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHHAYA SAYALA Telephone No. (703) 308-0196



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/04095

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EL-GHANNAM et al. Bioactive material template for in vitro synthesis of bone, Journal Biomedical Materials Research, 1995, Vol. 29, pages 359-370, see whole document.	1-38
Y	CANNAS et al. Biopiant Surfaces: Binding of fibronectin and fibroblast adhesion. Journal Orthopedic Research, 1988, Vol. 6, No. 1, pages 58-62.	1-38
Y	WO 95/14127 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 26 May 1995, especially claims.	1-38

# INTERNATIONAL SEARCH REPORT

International application No.  
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## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS

implant, substrate, ceramic, glass, bioactive, collagen, fibronectin, osteogenic, vitronectin.